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Flax-retting by polygalacturonase-containing enzyme mixtures and effects on fiber properties

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Abstract

Enzyme-retting of flax was accomplished via individual treatment with four polygalacturonase (PGase) containing solutions of various fungal sources and the resulting fibers were characterized. The retting solutions were equilibrated to contain 2.19 U of PGase activity as determined via a dinitrosalicylic acid (DNS) reducing sugar assay. As compared with the buffer control, treatment with the various enzyme solutions increased the yield of fine fibers. Treatment with *Aspergillus niger* PGase resulted in a 62% increase in fine fiber yield as compared with the buffer control and fiber strength did not statistically differ ($P \leq 0.05$) between these treatments. Retting via PGases of *Rhizopus* origin produced the weakest fibers. These results illustrate that the crude PGases differ in their ability to ret flax and that under the defined experimental conditions the *A. niger* PGase is a better retting agent. Light microscopy demonstrated the ability of all enzymes to separate fiber from shive and epidermal tissues. Enzyme profiles of the solutions were determined via viscometric assays. Pectinolytic activity was the predominant activity of all enzymes tested. Activity against carboxymethyl cellulose (CMC) was a minor component of all solutions except *A. niger* PGase for which no activity was detected. Published by Elsevier Science B.V.

Keywords: Flax; Polygalacturonase; Pectinase; Enzyme-retting; Fiber properties

1. Introduction

Traditionally, cellulosic fibers from flax (*Linum usitatissumum* L.) have been used primarily as a source of long fiber for the manufacture of linen. Short fiber or tow was a by-product of long fiber processing and is usually considered to be of less value than long-line fiber. Recently, however, interest in the use of short or 'cottonized' fibers for textiles and composites has grown, and further research involving flax fiber has been initiated (Akin et al., 2000, 2002a; Foulk et al., 2000, 2001).

Flax fibers develop from cells located within the phloem of the flax stem (Van Sumere, 1992). During maturation, the fiber cells differentiate from the phloem parenchyma by synthesizing successive layers of cellulose and pectins (Morvan et al., 1989). Furthermore, ultimate flax fibers are

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arranged in bundles which are separated by pectin-rich parenchyma cells. The pectic substances form an amorphous matrix which surrounds the cellulose fibers (Carpita and Gibeaut, 1993). Pectins are complex heteropolysaccharides with a main chain of α-1,4-linked galacturonan units, which can be esterified to varying degrees with a methoxyl group at the C-6 position (approximately 70%), *O*-acetylated, or substituted with xylose. The polygalacturonan chain may also contain some rhamnose residues which may be highly substituted with galactose or long-branched arabinose side chains (Sakai et al., 1993; De Vries et al., 1982). For use, flax fibers must be released from the pectic matrix and other cohesive substances which bind the fibers to the shive via cortex cells and the underlying secondary phloem (Chesson, 1978). This separation of fiber from non-fiber tissues is known as retting.

Traditionally, two types of retting have predominated, namely dew- and water-retting. Dew-retting largely relies on indigenous soil fungi to colonize the flax stems and to degrade pectin and hemicellulose by releasing polygalacturonase (PGase), xylanase and arabinase (Brown et al., 1986; Brown and Sharma, 1984). Resulting fibers are often coarse and of variable quality. Furthermore, retting by these means is limited to geographical regions with the appropriate weather conditions (Akin et al., 2000; Meijer et al., 1995; Van Sumere, 1992). Conversely, water-retting is performed in an aqueous environment, and anaerobic, pectinolytic bacteria are responsible for the decomposition of pectic substances and the subsequent release of flax fibers (Van Sumere, 1992; Chesson, 1978; Avrova, 1975; Osman et al., 1969; Allen, 1946). This process consistently yields high quality fibers (Van Sumere, 1992). However, use of water-retting is limited by environmental concerns as resulting effluents lead to serious pollution due to fermentation products (Van Sumere, 1992). Because of the deficiencies of dew- and water-retting, alternatives to these retting methods are being sought (Zhang et al., 2000; Meijer et al., 1995; Sharma and Van Sumere, 1992; Van Sumere, 1992).

Chemical and enzyme-retting are two such alternatives. Like dew- and water-retting, these methods free the fibers by disrupting the cohesive bonds which bind fiber and fiber bundles within the stem. In addition, retting by these means offers substantially more control compared with dew- and water-retting. Chemical treatments investigated include treatments with acid, base (Dujardin, 1948), surfactants, and chelators (Henriksson et al., 1998; Sharma, 1988). However, the fiber properties of chemical-retted flax can be unsatisfactory and may require large inputs of energy and generate costly wastes (Brühlmann et al., 2000; Van Sumere, 1992).

With enzyme-retting, flax stems are incubated in the presence of specific plant cell wall-degrading enzymes. Ideally, enzyme selection and treatment would result in hydrolysis of flax pectins and hemicellulases without damage to the cellulose fibers. In addition, by treating with specific enzymes or a specific enzyme mixture, consistently retted fibers would result (Van Sumere, 1992). Due to the structural complexity associated with the flax stem, it was originally suggested that a mixture of enzyme activities (e.g. pectinase, xylanase, and cellulase) would be needed to free the fibers (Van Sumere, 1992). Accordingly, two commercially available enzyme mixtures (Flaxzyme and SPS-ase 249) containing PGase, pectin lyase, hemicellulase, and cellulase were identified which were capable of retting flax (Anon, 1987; Sharma, 1987). However, Henriksson et al. (1999) successfully retted flax with a *Rhizopus oryzae* sb (incorrectly identified as *Rhizomucor pusillus*) culture filtrate, which lacked xylanase and mannanase activities, thereby suggesting that hemicellulases may not be necessary. The *R. oryzae* filtrate was shown to have high pectinase activity and further work implicated a strong (endo) PGase as the primary retting agent (Akin et al., 1999, 2001). Recently, Zhang et al. (2000) reported that PGase-induced degradation of low esterified pectin was the sole activity that correlated with the ability of enzymatic mixture to perform retting. Furthermore, these authors demonstrated that a purified (endo) PGase from *Aspergillus niger* was capable of retting flax. However, in this study the retting

abilities of the various enzymes and enzyme mixtures were estimated via small scaled Fried's Tests and not from the actual retting of flax. Therefore, to further emphasize the importance of PGase activity in flax-retting, we have performed enzymatic rettings using multiple PGase-containing preparations equated on the basis of their PGase activity. Specific non-PGase activities of the crude preparations were also determined and the properties of the resulting fibers compared.

2. Materials and methods

2.1. Materials

Mature 'Ariane' fiber flax was grown in the coastal plain region of South Carolina. After seed collection, stems were cut, dried, and crimped in preparation for retting (Akin et al., 2001; Foulk et al., 2000). Polygalacturonic acid (PGA) (P-3889) was prepared as 1% (wt./vol) solution in 50 mM sodium acetate buffer (pH 5.0).

2.2. Enzymes

The PGase-containing enzymes utilized in this study are shown in Table 1. Enzymes provided in liquid form (Viscozyme L., Novozymes, Franklin, NC) were used as provided (100%, vol/vol) and desiccated enzymes were prepared as 100% (wt./vol) solutions in 50 mM sodium acetate buffer (pH 5.0). Both served as sources for subsequent experiments.

Table 1
Experimental polygalacturonase-containing enzymes

Enzyme formulation, composition	Product No., Company
A. niger PGase, 100% (wt./vol)	31660, Serva Feinbiochemica GmbH, Germany
Rhizopus sp. PGase I, 100% (wt./vol)	P-4300, Sigma Chemical Co., St. Louis, MO
Rhizopus sp. PGase II, 100% (wt./vol)	76285, Fluka Chemie GmbH, Switzerland
Viscozyme L, 100% (vol/vol)	KTN02006, NovoNordisk Biochem, Franklin, NC

2.3. Enzyme assays

PGase activity was determined via the reducing sugar or dinitrosalicylic acid (DNS) assay with a glucose standard (Miller, 1959). To minimize reducing sugar background, Microcon YM-3 filters (Millipore, Bedford, MD) were used for buffer exchange and crude enzyme purification. Reaction mixtures contained 4 g l^{-1} of PGA and were incubated at 37 °C for 15 min. Absorbancy was determined via a microtitre plate reader (OD_{550}). Units of activity were defined as the amount (ml) of enzyme solution required to release 1 μmol of reducing sugar per min (μmol min^{-1} ml^{-1}).

Enzymatic activities against esterified pectin (approximately 90% esterified), pectin from citrus fruits, carboxymethyl cellulose (CMC), and oat spelt xylan were determined via viscometry with a Brookfield Laboratory DV-II + Digital Viscometer/Rheometer (Brookfield Engineering, Middleboro, MA). All substrates except xylan were prepared as 1% solutions in 50 mM sodium acetate buffer (pH 5.0) and added to achieve final reaction concentrations of 6.67 g l^{-1}. Xylan was prepared as a 2% solution in dH_2O and was added to achieve the concentration of 13.3 g l^{-1}. Enzymes (10%, vol/vol) with substrate were incubated in a 40 °C water bath for 15, 45, or 60 min. Viscosities of the resulting solutions were determined at 40 °C at pH 5.0 in 50 mM sodium acetate buffer at 60 rpm. Activity was estimated from the reduction in viscosity (ΔmPas). Units of activity were defined as the amount of enzyme required to reduce viscosity (mPas) by 1% per min (ΔmPas min^{-1} ml^{-1}). All assays were performed in duplicate ($n = 2$).

2.4. Flax-retting

Samples of crimped flax straw (50 g) were soaked for 1 min with frequent inversion in 200 ml (pH 5.0) of 2.5% Mayoquest (18 mM EDTA) (Callaway Chemical Co., Smyrna, GA) with appropriate concentrations of individual enzymes. Samples were then drained for 30 s, placed in ZipLock® bags and incubated at 40 °C. After 20 h, samples were rinsed with cold tap water (2 min) and allowed to air dry in an aerated hood. Dried

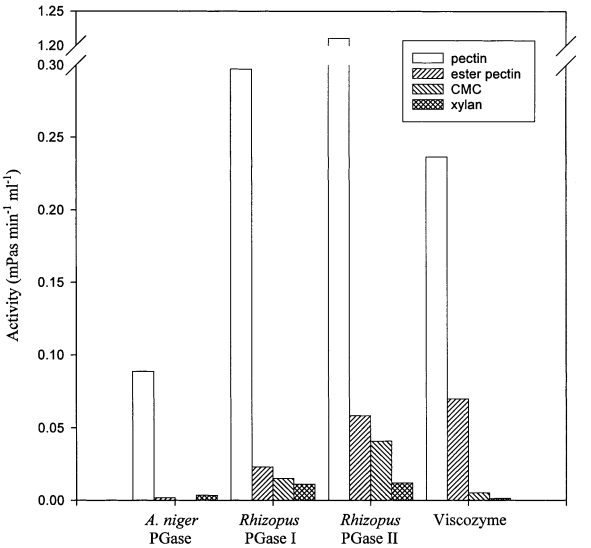

Fig. 1. PGase activities of enzymatic solutions.

samples were cleaned via three passes through a Shirley Analyzer (SDL America Inc., Charlotte, NC), which separated fine fiber from coarse fiber and shive. Resulting fine fiber was used to determine yields. Strength of Shirley-cleaned fibers was determined by Stelometer (Akin et al., 2001, 2002a; ASTM, 1997a). Fiber fineness was analyzed by micronaire modified to use 5 g substrate based on flax calibration standards (Akin et al., 2001, 2002a; ASTM, 1997b). Structural differences among retted fiber were analyzed by light microscopy.

3. Results

DNS assays were performed with PGA serving as the substrate. Results from the DNS assays demonstrated that the crude preparation Viscozyme L exhibited the highest PGase activity (4384 U) and that the PGases from *A. niger*, *Rhizopus* sp. I, and *Rhizopus* sp. II were 2759, 363, and 168 U, respectively (Fig. 1). Previous research in our laboratory has shown that effective retting can be achieved via 0.05% of Viscozyme L and 18 mM EDTA in the commercial product Mayoquest 200 (Akin et al., 2002a). If PGase activity is primarily responsible for retting, then it can be inferred that 2.19 U of PGase activity were responsible for releasing the fibers. Therefore, the experimental enzyme formulations were adjusted so that 2.19 U (PGase activity) of each enzyme preparation were used to ret 50 g samples of mature Ariane fiber flax.

Following enzyme-retting, fibers were isolated from the treated flax and fiber yields and properties were determined (Table 2). Treatment with the various enzymes resulted in a numerical increase of the yield of fine (i.e. Shirley-cleaned) fibers as compared with the buffer-treated control. However, only yields from the *A. niger* PGase- or Viscozyme L-treated flax were significantly higher ($P \leq 0.05$) than the buffer control, with *A. niger* PGase-treated flax yielding 62% more fine fibers

Table 2
Fiber yield and properties of flax enzyme-retted with various polygalacturonase-containing enzymes[a]

Enzyme[b]	Shirley-cleaned yield (%)[c]	Strength (g per tex)[c]	Elongation (%)[d]	Fineness (Mic)[e]
Buffer control	15.5 ± 2.3^{C}	43.6 ± 4.4^{A}	1.4 ± 0.2^{A}	8.0 ± 0^{A}
A. niger Pgase	24.9 ± 2.2^{A}	40.6 ± 1.9^{A}	$1.8 \pm 0.4^{A,B}$	$6.8 \pm 0.7^{B,C}$
Rhizopus sp. PGase I	17.1 ± 2.6^{C}	23.8 ± 1.5^{C}	$1.1 \pm 0.2^{B,C}$	$7.2 \pm 0.3^{A,B}$
Rhizopus sp. PGase II	$18.9 \pm 1.4^{B,C}$	20.4 ± 2.1^{C}	0.7 ± 0.4^{C}	6.4 ± 0.1^{C}
Viscozyme L.	$22.4 \pm 2.8^{A,B}$	32.2 ± 2.2^{B}	0.8 ± 0.1^{C}	$7.1 \pm 0.6^{B,C}$

[a] Triplicate 50-g samples of crimped flax (Ariane SC 99 late harvest) soaked 1 min in enzymes + 20 mM EDTA from Mayoquest 200 in water, pH 5.0, incubated at 40 °C for 20 h.

[b] Enzyme levels used that gave endopolygalacturonase activities equal to that in 0.05% v/v Viscozyme L.

[c] Fine fiber collected after three passes through a Shirley Analyzer.

[d] Average and standard deviation of three replicates, each replicate an average of six tests by Stelometer.

[e] Fineness determined by readings based on micronaire but modified using flax calibration standards (Institut Textile de France, Lille) and 5.0 g samples. Average and S.D. of three replicates, each replicate an average of two tests.

^{A,B,C}, values within columns with different letters differ at $P \leq 0.05$.

Table 3
Non-PGase enzymatic activities of polygalacturonase-containing enzymes

Enzyme	(ΔmPas min^{-1} ml^{-1})[a]			
	Citrus pectin	Esterified pectin	CMC	Xylan
A. niger Pgase	111.6	2.2	0	4.3
Rhizopus sp. PGase I	49.2	3.8	2.5	1.8
Rhizopus sp. PGase II	92.8	4.5	3.1	0.9
Viscozyme L	472.9	139.5	10.32	2.5

[a] Values represent means of duplicate treatments of substrate with enzyme.

than the buffer control. The strongest fibers were present in the buffer control which had not undergone enzymatic degradation. However, the fiber strength from *A. niger* PGase-treated flax was high and did not statistically differ ($P \leq 0.05$) from that of the control. Treatment of flax with either PGase of *Rhizopus* origin produced the weakest fibers. While significant differences were found in elongation, the range among samples was small and, therefore, probably limits the importance of these differences. Statistically, the finest fibers as determined by micronaire airflow resulted from treatment with Viscozyme L, *Rhizopus* PGase II, *A. niger* PGase and the coarsest from the buffer-treated control.

Light microscopy of fibers from the various treatments further supports these findings (Fig. 2a–e). Unretted control fibers (Fig. 2a) appear as large coarse fragments with a substantial amount of attached shive and epidermal tissue. In contrast, the enzyme-retted fibers (Fig. 2b–e) are predominated by mostly finer, shive-free fibers. Large fiber bundles can also be found among the enzyme retted samples, particularly with the *Rhizopus* sp. PGase I-treated (Fig. 2c) and Viscozyme L.-treated fibers (Fig. 2e).

To better understand the variations in retting efficiencies displayed by the tested enzymes and in their resulting fiber characteristics, viscometric assays were performed to determine enzyme profiles of the enzyme solutions used in the study. The enzyme solutions were tested for activity against PGA, citrus pectin, esterified pectin, CMC, and oat spelt xylan (Table 3). PGA at 1% exhibited only neglible differences in viscometry compared with buffer control and, therefore, results were not reported (data not shown). Among the activities tested via viscometry, pectinase (against citrus pectin) was the major activity associated with the enzyme solutions, and Viscozyme L contained $\simeq$4-fold the pectinase activity of the other enzymes. Viscozyme L also exhibited substantial activity against esterified pectin, while only minor activity against this substrate was evident among the other enzymes. CMCase, and xylanase were minor constituents of all solutions tested, except for *A. niger* PGase for which no CMCase was detected.

4. Discussion

Data suggest that retting which results in degradation of pectin around and between fiber bundles, can result strictly from the degradation of the unmethylated or low-esterified homogalacturonan regions which occur throughout the flax stem wall (Akin et al., 2001; Cabin-Flaman et al., 1993; Roland and Vian, 1981). Furthermore, Zhang et al. (2000), predicted via Fried's Test that retting efficiency and the ability to degrade these cell wall components were highly correlated (correlation coefficient = 0.99). As the degradation of low-esterified pectin is catalyzed by PGase, the PGase activity of a given enzyme preparation should give an indication of the solution's retting ability. The various PGase-containing enzymes, which were equated at 2.19 U of PGase activity, effectively retted 50 g flax samples in a recently developed enzyme-retting system (Akin et al., 2000).

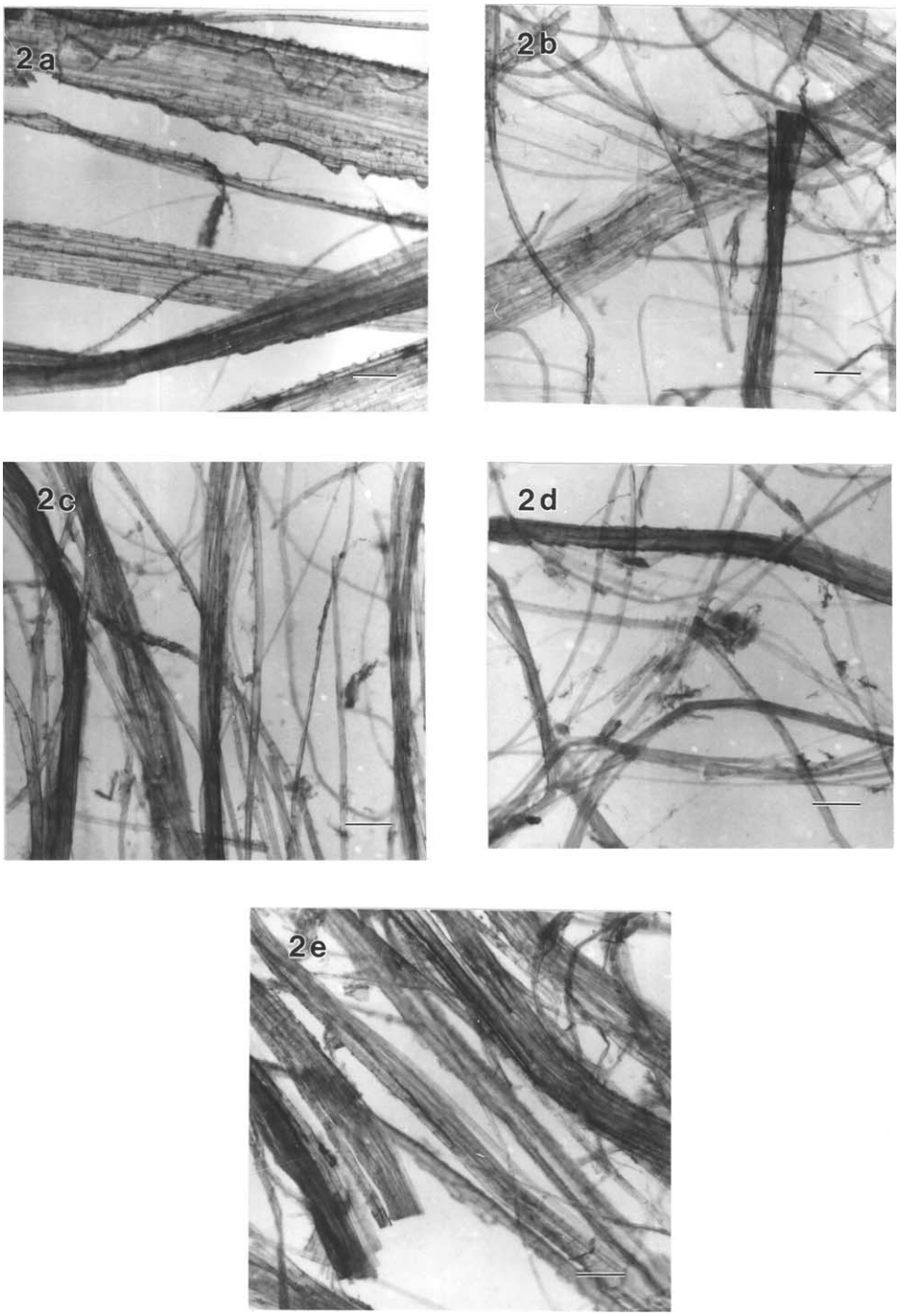

Fig. 2. Light microscopy of control, unretted (a), *A. niger* PGase-retted (b), *Rhizopus* PGase I-retted (c), *Rhizopus* PGase II-retted (d), and Viscozyme L-retted (e) 'Ariane' flax fiber. Bar = 200 μm.

A. niger PGase and Viscozyme L netted the highest fine fiber yields and were the strongest of all the enzyme treatments, with the *A. niger* PGase providing the highest quality textile fibers. Fineness and strength have important implications on the quality of the resulting yarn and strong, fine fibers are normally sought after for quality yarns (Van Sumere, 1992). As the PGase activity of all solutions was equated, this result could imply that the *A. niger* PGase contains a PGase more conducive to retting flax. Previously, it has been shown that PGases differ in substrate affinities and degradation patterns, which may affect both retting efficiency and fiber properties. In particular, numerous PGases have been isolated and characterized from *A. niger* which exhibit different preferences for substrate with regards to degree of methyl esterification (Kester et al., 1999). As pectins within the flax stem appear to be distributed according to type, it is possible that the PGase or PGases present in the *A. niger* preparation specifically target pectins which are strategic in releasing flax fibers (His et al., 1997; Cabin-Flaman et al., 1993; Jauneau et al., 1992; Knox et al., 1990). Resistance to PGase-inhibiting proteins, which are known to be associated with numerous plants, may have also influenced the results (Cook et al., 1999)

While the information presented here does not preclude the need for secondary enzymatic activities for the efficient retting of flax, it does further stress the importance of PGase in the process. Previous work has demonstrated the release of fibers from flax stems via treatment with a pure PGase on a small scale (Zhang et al., 2000). In addition, a PGase from *R. oryzae* has been implicated as a strong flax-retting agent (Akin et al., 1999, 2002b). Collectively, these works illustrate the intimate involvement of PGase in flax-retting and provide the basis for further work with this enzyme.

The differences encountered among the tested enzymes in retting ability and in the properties of the resulting fibers may also be attributed to the presence of additional enzyme (non-PGase) activities. Viscometric assays showed that in addition to PGase activity, the solutions also contain activities against citrus pectin and esterified pectin. Activity against citrus pectin is expected as regions of non-methoxylated polygalacturonic subunits are known to occur within this substrate (Zhan et al., 1998). However, little enzymatic cross reactivity should exist between the non-methoxylated PGA and the highly methoxylated esterified pectin. Previous work has shown that the occurrence of these methoxyl groups impedes PGase binding and thereby inhibits activity (Kester et al., 1999). Subsequently, degradation of esterified pectin may require a consortium of enzymes, namely pectin methyl esterase, PGase, and/or pectinolytic lyase.

Xylanase and CMCase were also exhibited among the enzyme solutions and are common contaminating activities associated with fungal enzymatic preparations. The relative proportions of the non-PGase activities varied among enzyme preparations. Furthermore, as the retting mixtures were formulated to contain equal amounts of PGase activity (2.19 U), their relative preponderance was further skewed (Fig. 3). For example, retting via the *Rhizopus* sp PGase II contained $\approx$4-fold excess enzymatic activity towards citrus pectin, while retting via *A. niger* PGase contained very little non-PGase activities.

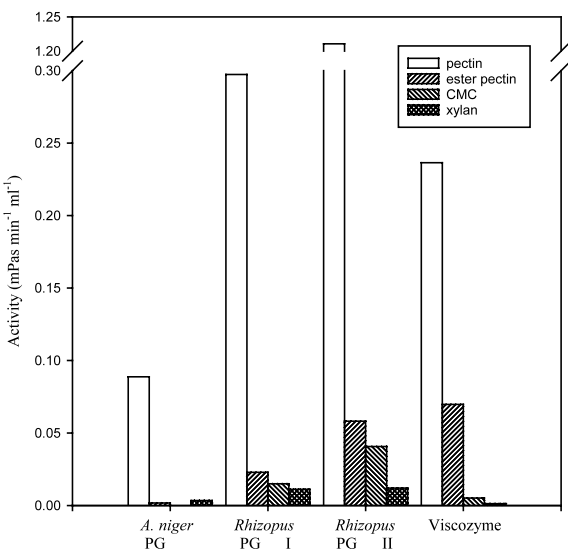

Fig. 3. Non-PGase enzymatic activities of retting solutions as determined via viscometric analysis (activity = mPas min^{-1} ml^{-1}).

During enzyme-retting, enzymes degrade the cell wall components surrounding the fibers. In addition, these enzymes may also act on the fibers and affect their resulting properties. Within this study, the *A. niger* PGase treatment which contained minimal non-PGase activities yielded high quality fibers that were among the finest and strongest produced. In contrast, fibers resulting from the other treatments containing increased non-PGase activities were normally of lower quality exhibiting less strength and fineness. It is possible that the differences in fiber characteristics can be attributed to non-PGase activities. For example, in this study, CMCase activity appears to inversely effect fiber strength.

5. Conclusion

Collectively, these results illustrate that the crude PGases differ in their ability to ret flax and that under the defined experimental conditions the *A. niger* PGase is a better retting agent. While all enzymes tested were capable of releasing the flax fibers from the flax stem, *A. niger* treatment produced the strongest and finest fibers. The *A. niger* preparation also contained minimal non-PGase activities lending further support against the need for multiple enzymatic activities to ret flax. As the enzymes used in this study were crude preparations, little inference can be made about their associative PGases. Furthermore, this work also suggests the importance of the use of a non-cellulolytic enzyme solution for the production of strong fibers.

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